NUCB2/nesfatin-1: A New Adipokine Expressed in Human and Murine Chondrocytes with Pro-Inflammatory Properties, An In Vitro Study

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ABSTRACT: Nesfatin-1 is a recently discovered satiety-inducing adipokine identified in hypothalamic regions that regulates energy balance. So far, no data exist on NUCB2/nesfatin-1 localization in human and murine chondrocytes. Here, we therefore investigated NUCB2/nesfatin-1 gene and protein expression in human and murine chondrocytes and the effect of nesfatin-1 on pro-inflammatory cytokines expression. Peptide localization was performed by laser confocal microscopy, NUCB2 mRNA expression was studied by RT-PCR and protein secretion was measured by XMap technology and Western blot analysis. First, we demonstrated cytoplasmic localization of NUCB2/nesfatin-1 peptide in both human and murine chondrocytes. We present evidence that both mRNA and protein expression of NUCB2 were increased during the differentiation of ATDC5 murine chondrocyte cell line. Furthermore, we demonstrated that nesfatin-1 induces IL-6 and MIP-1α mRNA expression and protein secretion in ATDC-5 cells challenged with IL-1, and also increases COX-2 mRNA expression in these cells. Finally, nesfatin-1 provoked a clear induction of pro-inflammatory agents, such as COX-2, IL-8, IL-6, and MIP-1α in human primary chondrocytes from OA patients. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:653–660, 2014.

Keywords: Nesfatin-1; NUCB2; cytokines; chondrocytes; inflammation

Nesfatin-1 is a recently identified satiety-inducing adipokine found in hypothalamic regions that regulates energy balance.[1] It is a peptide derived from its precursor protein, non-esterified fatty acid/nucleobindin 2 (NUCB2) that is composed by a signal peptide of 24 amino acids and a protein structure containing 396 amino acids. The homology of the amino acids sequence of NUCB2 is highly conserved in humans, mice and rats. Post-translational processing of NUCB2 by prohormone convertase (PC) produces three cleavage products: nesfatin-1 (1–82), nesfatin-2 (85–163), and nesfatin-3 (166–396). The PC recognition sites within NUCB2 are highly conserved and the cleavage is predicted to yield the 82, 79, and 231 amino acid peptides, nesfatin-1, nesfatin-2, and nesfatin-3, respectively. To date, no biological role has been attributed specifically to nesfatin-2 and nesfatin-3. Several studies indicated a potential implication of nesfatin-1, the N-terminal fragment of NUCB2, as an anorexigenic molecule. In fact, NUCB2/nesfatin-1 is abundantly expressed in several regions of the hypothalamus that play key roles in controlling food intake.[2] In addition, it has been showed that NUCB2/nesfatin-1 was able to reduce food intake in rodents when administrated either centrally or peripherally.[1,3] NUCB2/nesfatin-1 was expressed in gastric endocrine cells[4] and recently the group of Ramanjaneya observed that another potential source of peripheral nesfatin-1 is adipose tissue, preferentially the subcutaneous tissue.[5] Recent studies showed the expression of NUCB2/nesfatin-1 mRNA in other peripheral tissues including pancreas, islets, heart, stomach, and also spinal cord.[4,6,7] Very recently, NUCB2/nesfatin-1 has been identified also in human and murine cardiomyocytes where it is able to induce glucose uptake and the mobilization of the glucose transporter GLUT-4.[8] During the writing of this article, Li et al.[9] demonstrated that nesfatin-1 was able to increase bone mineral density of ovariectomized rats, suggesting a pro-osteogenic activity of this adipokine that would represent a valuable treatment of bone metabolic diseases such as osteoporosis. NUCB2/nesfatin-1 has strong similarities in terms of metabolic actions with other members of adipokine superfamily, such as leptin, adiponectin, and lipocalin-2. These factors have been recently identified, by our group and others, as active players in the regulation of physiological and pathological processes, such as inflammation and immune response in whole joint tissues and in rheumatic diseases, such as osteoarthritis (OA) and/or rheumatoid arthritis (RA).[10–14] Recent evidence suggests that central nesfatin-expressing neurons are sensitive to peripheral inflammatory stimuli and could account for the reduction in food intake.[15] In addition, nesfatin-1 has been supposed to play a role in the modulation of inflammatory response in the central nervous system.[16,17]

To the best of our knowledge, no data exist on NUCB2/nesfatin-1 localization in human and murine

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chondrocytes and its expression in these cells. We therefore investigated NUCB2/nesfatin-1 gene and protein expression in human and murine chondrocytes and we have also analyzed the effects of nesfatin-1 on induction of pro-inflammatory factors. Finally, NUCB2 modulation after treatments with classic pro-inflammatory cytokines such as IL-1 and TNF-alpha has been also explored.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), human transferrin, sodium selenite were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, l-glutamine and antibiotics were purchased from Lonza (Verviers, Belgium). Nesfatin-1 (1–82) was purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany). Immuno-Pure normal goat serum and TOPRO-3 iodide from Invitrogen (Carlsbad, CA). Mowiol was obtained from Calbiochem (Darmstadt, Germany).

Cell Culture and Differentiation

The murine chondrogenic cell line ATDC-5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM–Ham's F-12 medium supplemented with 5% FBS, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Chondrogenic ATDC-5 cells were differentiated into mature and hypertrophic chondrocytes, as previously described.[18] Briefly, these cells undergo a late differentiation phase, becoming hypertrophic, calcifying chondrocytes that synthesize type X collagen and osteopontin, a marker of terminal chondrocyte differentiation. The differentiation from Days 0 to 21 was further evidenced by sequential increases in type II collagen, aggrecan, and type X collagen mRNAs. The early and mature chondrocyte marker type II collagen was expressed in undifferentiated ATDC5 cells; the level began to increase at Day 3, peaked at Days 7–10 and gradually declined after Day 15. The expression profile of aggrecan mimicked that of type II collagen but with a slight delay of a couple of days. The decline in expression of both chondrocyte markers coincided with the onset of late-stage chondrocyte differentiation. The expression of the hypertrophic chondrocyte marker type X collagen began

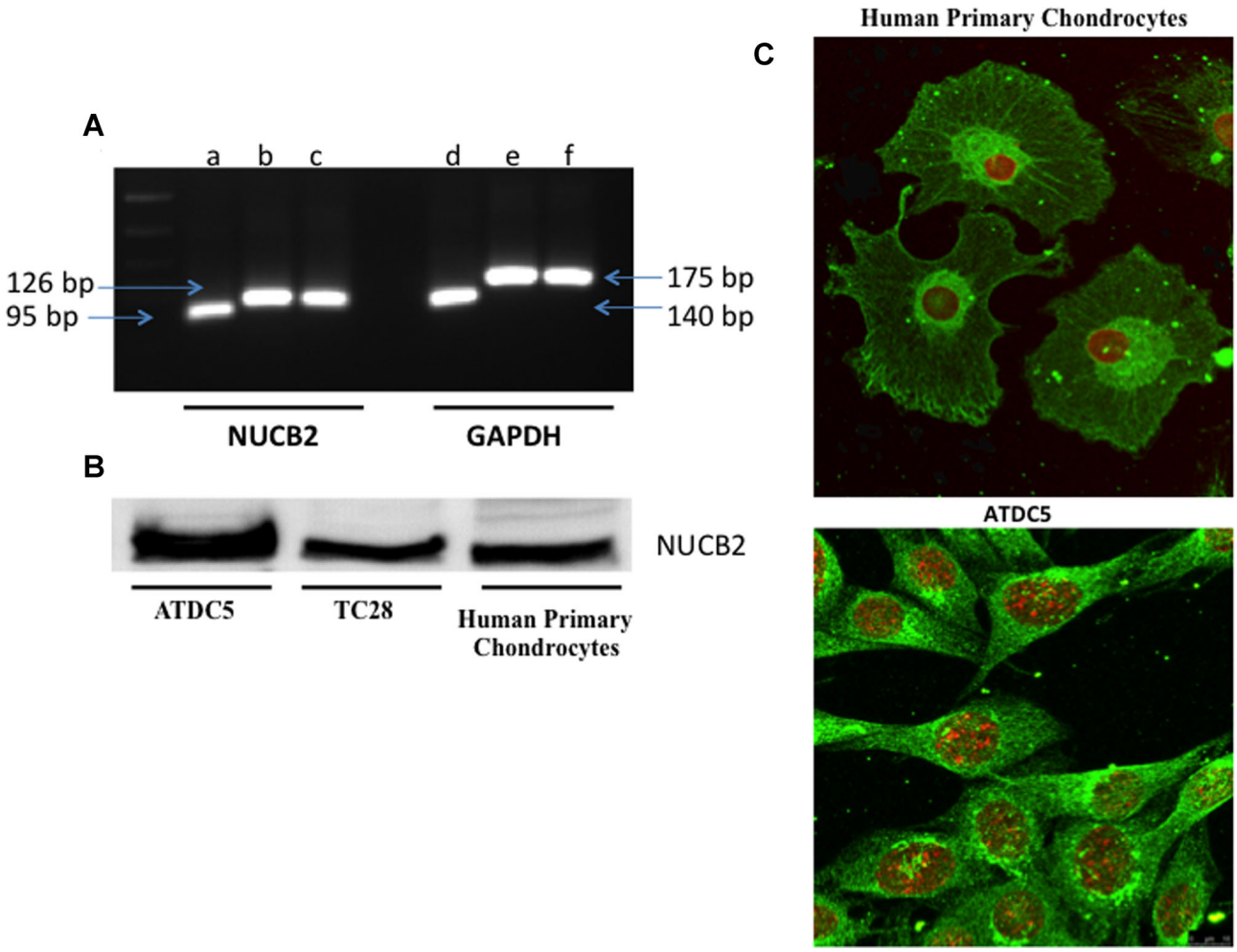

Figure 1. Basal expression evaluated by RT-PCR of NUCB2 mRNA (panel A) in human primary chondrocytes (line c), T/C-28a2 human cell line (line b) and ATDC-5 murine chondrocytes (line a). Amplicons were electrophoresed on 1.8% agarose gel, stained with ethidium bromide and visualized with a high definition CCD camera. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression is also shown (lines d, e and f). NUCB2 protein expression was reported in (panel B). Cytoplasmic localization of NUCB2/nesfatin-1 in human primary chondrocytes and ATDC-5 by laser confocal microscopy were showed in (panel C).

at Days 12 and 13. The expression patterns of these early and late chondrocyte markers were consistent with previous findings in ATDC5 cells regarding in vivo chondrocyte differentiation. We do not illustrate findings regarding the differentiation of ATDC5 cells because they are extensively reported in literature.[19] T/C-28A2 human immortalized chondrocytes were a kind gift of Dr. Mary B. Goldring (Hospital for Special Surgery, NYC, USA). Primary chondrocytes were harvested from human OA articular cartilage samples obtained from articular joints of patients undergoing total knee replacement surgery as previously described.[11,14,18] All experiments were performed in the first passage of culture.

Cell Treatments, Protein Extraction, and Western Blot Analysis

ATDC-5 were plated at a density of 3×10^5 cells/well in 6-well plates and incubated with IL-1 0.05 ng/ml and nesfatin-1 1 μM during 24 h at 37°C in serum-free media. Human primary chondrocytes were seeded at a density of 3×10^5 cells/well in 6-well plates and treated with nesfatin-1 300 nM during 24 h at 37°C in serum-free media.

Proteins were extracted using a NucleoSpin kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. SDS-PAGE and blotting procedure were carried on as previously described.[20] Immunoblots were incubated with the appropriate antibody (anti-nesfatin-1 (1–82) antibody, Phoenix Pharmaceuticals) and visualized with an Immobilon Western Detection kit (Millipore, MA) using horseradish peroxidase-labeled secondary antibody. To confirm equal loading in each sample, the membranes were stripped in glycine buffer at pH 2 and re-blotted with anti-β-actin antibody (Sigma). Images were captured and analyzed with an EC3 imaging system (UVP).

mRNA Isolation and RT-PCR

Murine and human MIP-1α, murine and human IL-6, murine and human COX2 and human IL-8 mRNA levels were determined using SYBR Green-based quantitative PCR. Murine and human NUCB2 mRNA levels were determined using FAM Green-based quantitative PCR. RNA was extracted using a NucleoSpin kit (Macherey-Nagel), according to the manufacturer's instructions. For relative quantification, we performed an RT reaction with a Thermo Scientific Verso cDNA Synthesis Kit (42°C for 30 min, followed by an incubation at 95°C for 2 min). RT-PCR was performed in a Stratagene MX3005P thermal cycler using a standard protocol (95°C for 10 min followed by 40 cycles for 15 s of denaturation at 95°C and 1 min annealing/extension at 60°C), a SABiosciences Master Mix and specific primers (for mouse MIP-1α, 112 bp, PPM02949E, reference position 436, GenBank accession no. NM_011337.2; for mouse IL-6, 178 bp, PPM03015A, reference position 73, GenBank accession no. NM_031168.1; for mouse GAPDH, 140 bp, PPM02946E, reference position 309–328, GenBank accession no. NM_008084.2; for human MIP-1α, 114 bp, PPH00566E, reference position 124, GenBank accession no. NM_002983.2; for human GAPDH, 175 bp, PPH00150E, reference position 1287–1310, GenBank accession no. NM_002046.3; for human IL-6, 160 bp, PPH00560B, reference position 755, GenBank accession no. NM_000600.3; for mouse COX-2 135 bp, PPM03647E, reference position 955, GenBank accession no. NM_292547; for human COX-2, 63 pb, PPH01136F, reference position 1502, GenBank accession no. NM_000963.2; for human IL-8, 126 pb PPH00568A, reference position 326, GenBank accession no. NM_000584.3). Murine NUCB2 was from SOLARIS, Thermo Scientific. Results of comparative RT-PCR experiments were analyzed using MxPro software (Stratagene, CA).

Laser Confocal Microscopy

ATDC-5 cells and human primary chondrocytes have been seeded at a density of 1×10^5 cells/well on a coverslip in 24-well plates in cultured media. After adhesion, we have removed cultured media and we have added formaldehyde 4% in PBS (phosphate buffered saline) during 20 min at room temperature. After three washes with PBS we have blocked non-specific binding sites with BSA/PBT-T (0,2% BSA; Tween-20; Triton-100; serum 5%; PBS 1X) during 30 min and next we have incubated with anti-nesfatin antibody 1:500 overnight at 4°C in blocking solution. Next day, we have washing four times with PBS 1X and we have added secondary antibody Cy™ 3-conjugated affinipure donkey anti-rabbit IgG (H+L) 1:500 45 min at 37°C in blocking solution. After four washes with PBS, we have added TOPRO-3 1:500 10 min in darkness at room temperature, next we have washed three times in PBS and we have mounted samples in slides using Mowiol. The next day we have acquired images by LEICA DMIRE2 confocal microscopy using a 63X objective, pixel resolution 512×512, scan speed 400 Hz. Negative controls were performed with non-specific rabbit IgG primary antibody obtaining no fluorescence signals (data not shown).

BioPlex Assay

BioPlex Pro Mouse cytokine (BioRad, Hercules, CA) assay have been performed to evaluate the effect of nesfatin-1 on inflammatory cytokines IL-6 and MIP-1α in ATDC-5 cells challenged with IL-1. Data have been analyzed with the BioPlex Manager 6.1 software generating 5-para meter logistic curve fit.

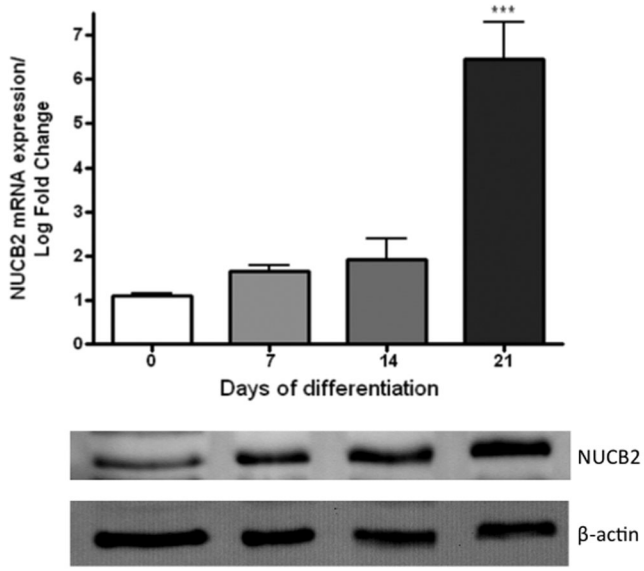

Figure 2. NUCB2 mRNA expression during ATDC-5 differentiation after 7, 14, and 21 days. Values are the mean ± SEM of at least 4 independent experiments. $^{***}p < 0.001$ vs. Control. Cell lysates underwent western blot analysis using NUCB2/nesfatin-1 antibody. β-actin was used as a loading control.

Statistical Analysis

Data are reported as the mean ± SEM of at least three independent experiments, each with at least three independent observations. Statistical analysis was performed using analysis of variance followed by the Student-Newman-Keuls test or Bonferroni multiple comparison test using the Prism computerized package (GraphPad Software, La Jolla, CA). p values less than 0.05 were considered significant.

RESULTS

NUCB2/Nesfatin-1 Basal Expression in Murine and Human Chondrocytes

NUCB2 mRNA expression was detected in both mouse and human chondrocytes. As shown in Figure 1A, RT-PCR analysis showed a high expression of NUCB2 in human primary chondrocytes (line c), in a human immortalised chondrocyte cell line T/C-28a2 (line b), and ATDC-5 murine chondrocytes (line a). Furthermore, western blot analysis confirmed NUCB2/nesfatin-1 protein expression in these cells (Fig. 1B). Finally, by laser confocal microscopy, we have demonstrated cytoplasmic localization of NUCB2/nesfatin-1 in both human and murine chondrocytes (Fig. 1C).

NUCB2 mRNA and Protein Production During ATDC-5 Differentiation

As shown in Figure 2, NUCB2 mRNA expression increased during the process of differentiation of ATDC-5 cells. This increase is highly significant after 7, 14, and 21 days of differentiation in comparison to undifferentiated cells (Day 0) (Fig. 2 upper panel). This effect was also evaluated in terms of protein expression as reported in Figure 2 (lower panel).

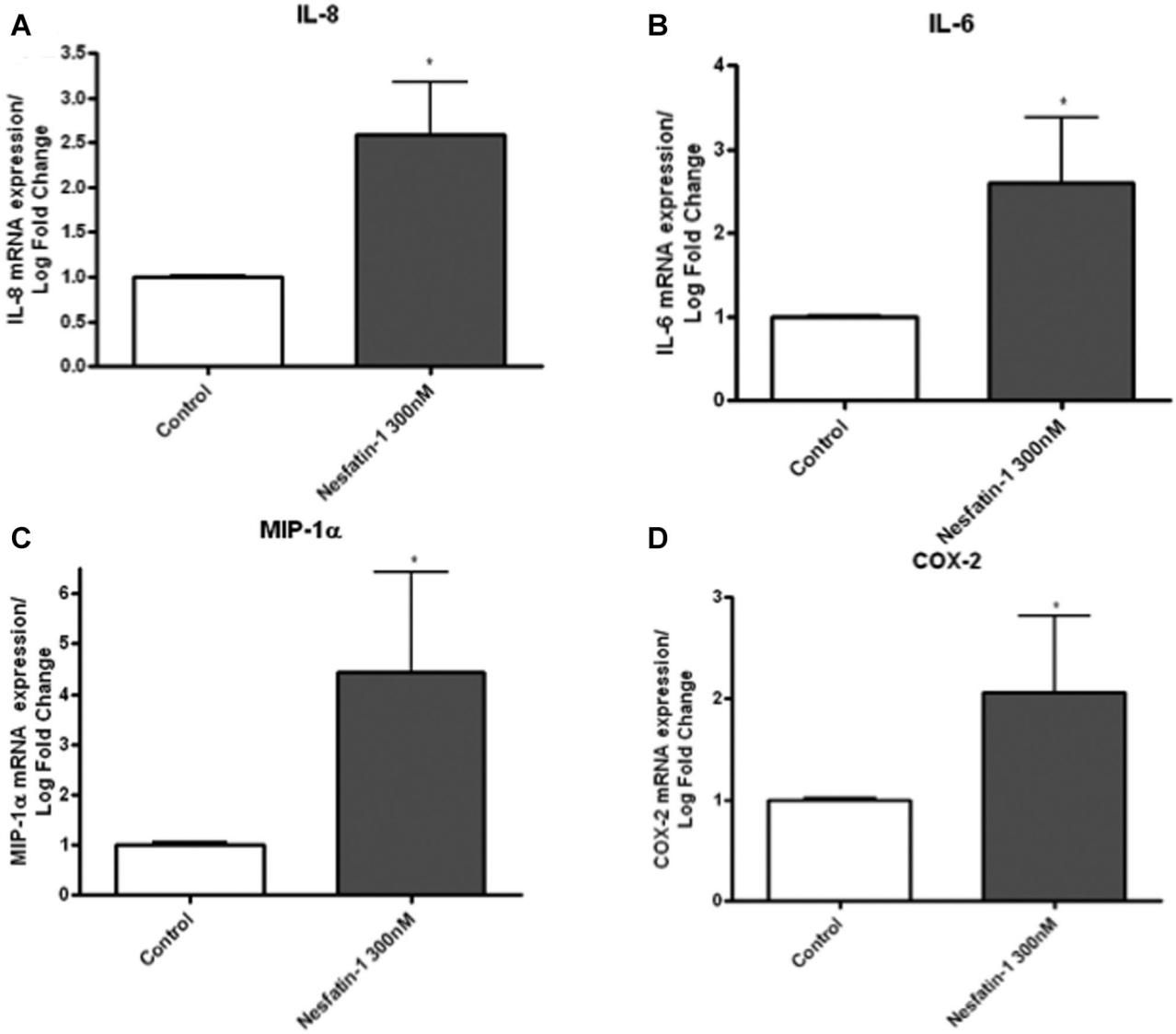

Figure 3. Human mRNA expression of IL-8 (A), IL-6 (B), MIP-1α (C), and COX-2 (D) after treatment with nesfatin-1 300 nM during 24 h in human primary chondrocytes from OA patients. Values are the mean ± SEM of at least 4 independent experiments. $^{*}p < 0.05$ vs. Control.

Nesfatin-1 Induces Pro-Inflammatory Mediators in Human Primary Chondrocytes from OA Patients

As showed in Figure 3, nesfatin-1 significantly increased IL-8, IL-6, COX-2, and MIP-1α mRNA expression in human primary chondrocytes ($p < 0.05$). Nesfatin-1, in OA human primary chondrocytes, is able to induce pro-inflammatory cytokines.

Effect of IL-1 and TNF-α on NUCB2 mRNA and Protein Expression in ATDC-5 Differentiated Cells and Human Primary Chondrocytes from OA Patients

As shown in Figure 4A, ATDC-5 differentiated (14 days) cells treated with different doses of IL-1 (0.1 and 0.5 ng/ml) during 24 h showed a clear and significant increase in NUCB2 mRNA expression as well as protein synthesis. A similar effect was observed when cells were stimulated with TNF-α (0.1, 1. and 10 ng/ml; Fig. 4B) during 24 h. In addition, as shown in Figure 4C NUCB2 protein expression is increased also in OA human primary chondrocytes after 24 h stimulation with IL-1 (0.1 and 0.5 ng/ml) and TNF-alpha (1 and 10 ng/ml; Fig. 4D).

Nesfatin-1 Potentiates the Induction of MIP-1α and IL-6 in ATDC-5 Chondrocytes Challenged with IL-1

To gain further insight into the potential pro-inflammatory activity of nesfatin-1 we have evaluated the induction of two relevant mediators of inflammation in cultured ATDC-5 stimulated with nesfatin-1 alone or in combination with IL-1. As shown in Figure 5A, nesfatin-1 alone (at a dose of 1 μM) was not able to induce IL-6 in ATDC-5 cells. On the contrary, IL-1 (at a dose of 0.05 ng/ml) was a frank inducer of IL-6 in ATDC-5 cultured cells. When the cells have been stimulated with a combination of IL-1 and nesfatin-1, the expression of IL-6 was significantly higher than in the cells stimulated with IL-1 and nesfatin-1 alone. Noteworthy, nesfatin-1 was also able to exert a similar activity on other inflammatory mediators such as MIP-1α (Fig. 5B). These results were confirmed also in terms of mRNA expression (Fig. 5C and D). To note,

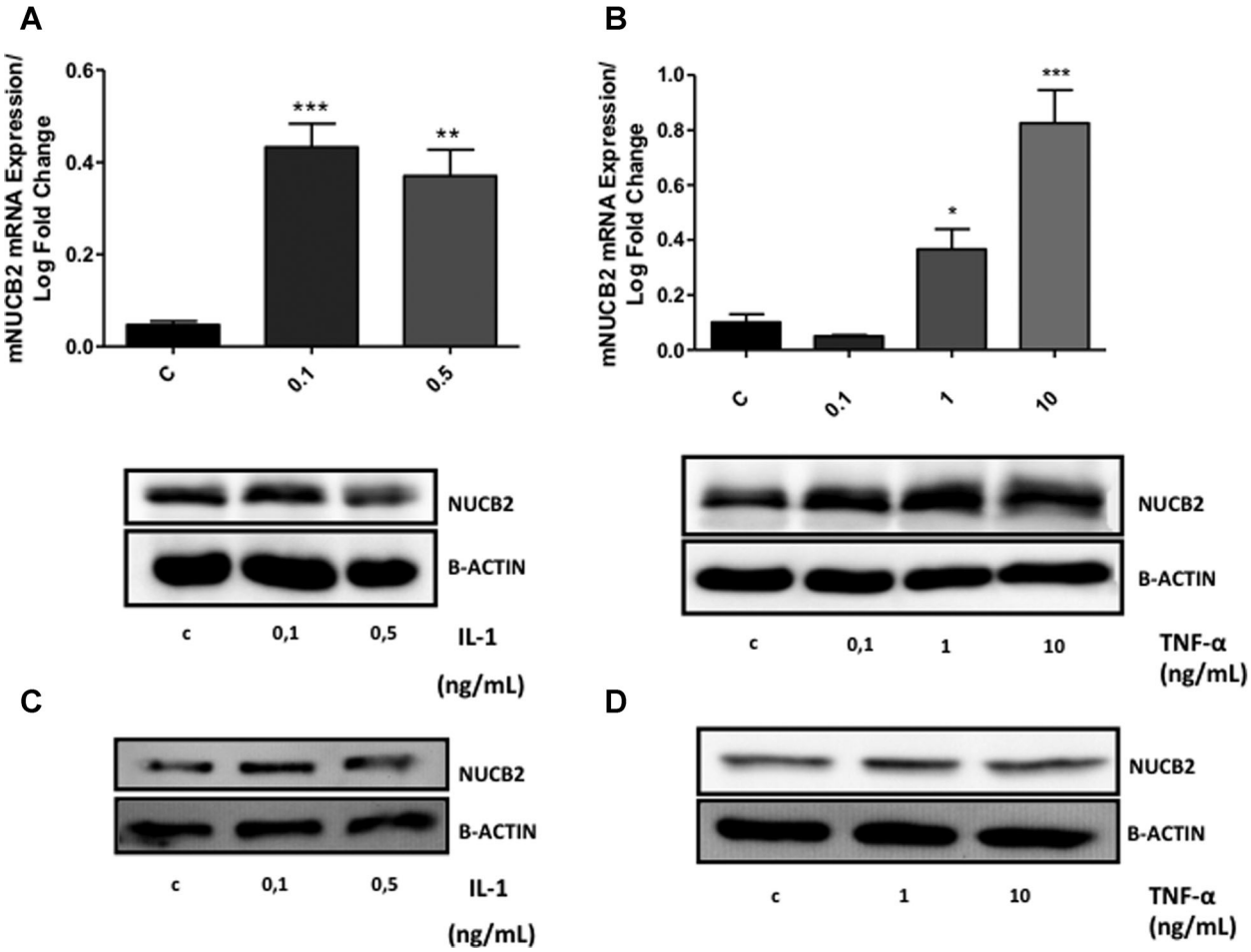

Figure 4. Mouse NUCB2 mRNA and protein expression after IL-1 (0.1 and 0.5 ng/ml) treatment for 24 h in ATDC-5 differentiated cells (14 days) cells (panel A). Mouse NUCB2 mRNA and protein expression after TNF-α (0.1, 1, and 10 ng/ml) treatment for 24 h in ATDC-5 differentiated (14 days) cells (panel B). Human NUCB2 protein expression after IL-1 (0.1 and 0.5 ng/ml; Panel C), and TNF-α (1 and 10 ng/ml; Panel D) treatment for 24 h in human primary chondrocytes from OA patients. β-actin was used as a loading control. Values are the mean ± SEM of at least 4 independent experiments. $^{***}p < 0.001$ vs. Control; $^{**}p < 0.01$ vs. Control; $^{*}p < 0.05$ vs. Control.

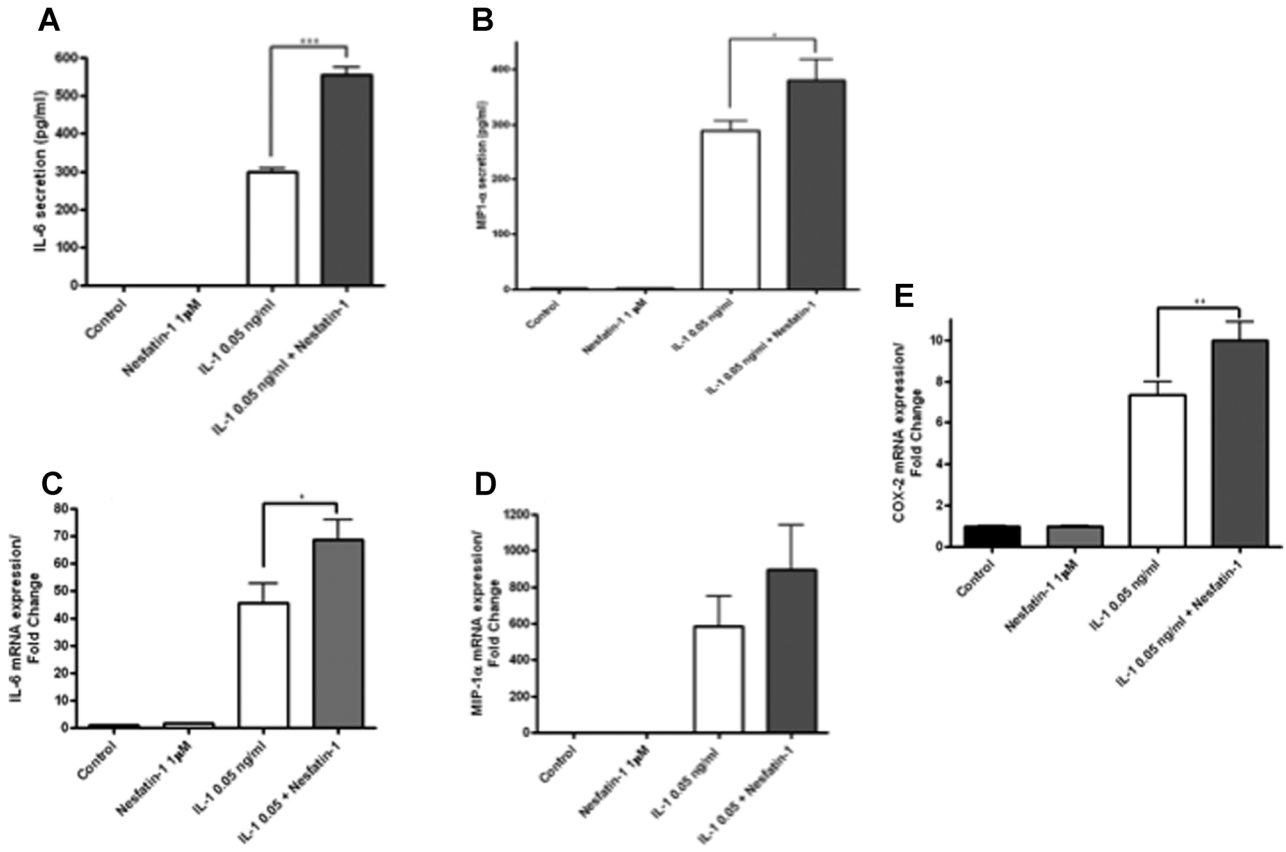

Figure 5. Mouse IL-6 protein secretion (panel A) and mRNA expression (panel C) after treatment with nesfatin-1 (1 μM) alone or in combination with IL-1 0.05 ng/ml during 24 h. Mouse MIP-1α protein secretion (panel B) and mRNA expression (panel D) after treatment with nesfatin-1 (1 μM) alone or in combination with IL-1 0.05 ng/ml during 24 h. Mouse COX-2 mRNA expression (panel E) after treatment with nesfatin-1 1 μM alone or in combination with IL-1 0.05 ng/ml during 24 h. Values are the mean ± SEM of at least 3 independent experiments. $^{***}p < 0.001$ vs. Control; $^{**}p < 0.01$ vs. Control; $^{*}p < 0.05$ vs. Control.

nesfatin-1 was also able to potentiate COX-2 mRNA expression in ATDC-5 cells challenged with IL-1 (Fig. 5E).

DISCUSSION

NUCB2/nesfatin-1 has been identified for the first time in the hypothalamus as a factor that regulates energy balance. However, peripheral production of this peptide has been also reported. In fact, NUCB2 mRNA was expressed in gastric endocrine cells[4] but also in other peripheral tissues including pancreas, heart and spinal cord.[6,7] Very recently, the group of Ramanjaneya[5] showed that human and murine adipose tissue is able to produce NUCB2/nesfatin-1, suggesting that this anorexigenic molecule is a novel adipokine. Nesfatin-1 protein levels are high in diet-induced obese mice, and human plasma nesfatin-1 correlated positively with increasing BMI. Some lines of evidence suggest that members of adipokine superfamily, such as leptin, adiponectin, lipocalin-2, and many other, exert relevant actions as key players of the complex network of soluble mediators involved in the pathophysiology of cartilage degenerative diseases. Thus, we have examined NUCB2/nesfatin-1 expression and secretion in these cells. To the best of our knowledge, this is the first time that, NUCB2 mRNA and protein expression is detected in human primary chondrocytes and murine ATDC-5 chondrocytes. Human and murine chondrocytes showed cytoplasmic localization of NUCB2/nesfatin-1. We also showed, for the first time, that NUCB2 mRNA and protein expression was increased during the process of ATDC-5 cells into mature chondrocytes. These data suggested that this peptide might play a role in cartilage maturation and in the complex mechanisms of chondrocyte development and differentiation. The strong significant increase, as differentiation progressed, went in parallel with formation of cartilage nodules and enhancement of mineralization, characteristic signs of differentiation of ATDC-5 cells. Together, these data suggest that NUCB2/nesfatin-1 might be a marker of late-phase chondrogenic differentiation and might affect endochondral ossification. To this regard, and possibly in agreement with data reported by our group, Li et al.[9] reported that nesfatin-1 promotes osteogenesis by increasing bone mineral density in ovariectomized rats. To note, nesfatin-1 also increased mineralization in MC3T3

pre-osteoblastic cells and encumbered osteoclastic differentiation of RAW 264.7 macrophages.

Apart from the first time report of the constitutive expression of NUCB2/nesfatin-1 in chondrocytes and its modulation along chondrocyte differentiation, our study makes another novel observation, that nesfatin-1 is able to significantly induce chemotactic and pro-inflammatory mediators such as IL-8, MIP-1α, IL-6, and COX-2. These factors are also induced by other adipokines (i.e., leptin and adiponectin in OA chondrocyte) suggesting that nesfatin-1 may participate, together with other previously described adipokines, in the pathogenesis and/or progression of inflammatory complications of cartilage degenerative diseases. To note, in ATDC-5 cells nesfatin-1 alone is not able to modulate neither IL-6 nor MIP-1α and COX-2. However, when cells were stimulated with a combination of nesfatin-1 and IL-1, a clear potentiation of response was observed. This synergy and/or potentiation, is not new in the adipokine superfamily. Actually, other adipokines (i.e., leptin) showed similar properties, being inactive alone but very active working in synergy with other cytokines.[11] The apparent discrepancy in the way of response between human and murine ATDC-5 cells is possibly due to the fact that OA chondrocytes can be considered as cells whose response is influenced by long term exposition to the inflammatory milieu existing in OA. On the contrary, ATDC-5 cells, when stimulated with nesfatin-1 alone, can be considered as cells without any previous exposition to inflammatory environment. In agreement with observation in human OA cells, there is the evidence that in ATDC-5 cells challenged with IL-1, a classic pro-inflammatory cytokine, nesfatin-1 is able to increase the inflammatory response in terms of increased synthesis and expression of chemotactic factors (IL-8, MIP-1α), cytokines (IL-6), and prostanoids (COX-2).

Regarding the influence of inflammatory milieu, another novel aspect of our results is the expression profile of NUCB2/nesfatin-1 in cells treated with IL-1 and TNF-α, two classic cytokines that are involved in OA and RA. This finding indicates that IL-1 and TNF-α, released from joint tissue, are involved from one side in the induction of pro-inflammatory mediators and proteinase that promote the destruction of cartilage matrix, as widely reported in the literature. On the other hand, these cytokines may alter constitutive expression of nesfatin-1 in chondrocytes that, in an autocrine or paracrine manner may perpetuate the inflammatory response by modulating the synthesis and or expression of chemotactic factors, cytokines, or prostanoids.

In conclusion, our data show, for the first time, that NUCB2/nesfatin-1 is synthesized and produced by human and murine chondrocytes. In addition, NUCB2/nesfatin-1 production was increased during the chondrocyte differentiation process and after the stimulation with pro-inflammatory mediators such as IL-1 and TNF-α. Furthermore, nesfatin-1 induces pro-inflammatory cytokines alone in OA human primary chondrocytes and in combination with IL-1 in murine chondrocytes. Our present study revealed novel basis for understanding the mechanism/s by which novel adipokines, such as nesfatin, may contribute to the function and regulation of chondrocyte pathophysiology.

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